

**PROGRESSIONAL CONSTRUCTION OF FREE STANDING
PERFUSABLE MICROVASCULATURE**

By

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A thesis submitted to Johns Hopkins University in conformity with the requirements for
the degree of Master of Science in Engineering

Baltimore, Maryland

April, 2015

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Abstract

Microvasculature is essential to connect main blood vessels to smaller capillary networks throughout the body. As with all vasculature, these structures can be impaired due to disease or injury, and their reconstruction is imperative to ensure the patient's health. However, most regeneration studies to date have focused on inducing or engineering either small capillary beds or large arteries and veins. Previously we developed a system to study microvasculature development guided on electrospun fibrin hydrogel microfibers. The focus of this work was to utilize this biomaterial to develop freestanding perfusable microvascular structures, a feat that has not been achieved to date. Progenitor endothelial cells and vascular smooth muscle cells were seeded onto microfibers to create a robust cell layer before applying a degradation treatment to remove the microfiber core, creating a perusable structure. We used this seeding protocol on three different set ups throughout the study to create our microvascular structures. The first set up relied on the scaffold using a frame for support. The scaffolds on frames were used to test if the cell layers would be damaged by the degradation treatments, which were then used to achieve luminal formation. When lumen formation was created without damage to the cell layers, we conducted the second set of experiments in two different devices. These devices allowed the scaffold to be freestanding and to support the growth of a robust cell layer. However, full degradation of the scaffold could not be obtained on cellularized microfibers due to a thick cell layer that prevented plasmin diffusion. Therefore, the shape of the microfibers was altered to allow direct perfusion via a hollow structure. With the use of direct perfusion, the hollow microfibers allowed for a cellular structure to grow with a distinct lumen. This engineered microvasculature has the

potential of creating patient specific perusable structures for cell therapy and drug delivery testing.

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Committee: Dr. Sharon Gerecht and Dr. Hai-Quan Mao

Acknowledgments

First and foremost, I would like to thank my parents, grandmother, and best friends who have been the best support group I could have ever imagined throughout my undergraduate and graduate studies. They have taught me to always keep an open mind and that hard work would help me achieve my goals in life.

Secondly, I would like to thank Dr. Sharon Gerecht, who has been a wonderful mentor and has given me the chance to explore the world of science in a new perspective. I am so grateful that she allowed me to join her laboratory my sophomore year to allow me the chance to evolve my passion for research. I could not have asked for a better advisor, with her guidance I have gained a strong work ethic and dedication to the projects that I had the pleasure of working on.

Many, many thanks to each of the members of the Gerecht Lab, who have inspired many new ideas to help understand and explore vascular tissue engineering over the past four years: Dr. Abigail Hielscher, Dr. Kyung Min Park, Dr. Xin Yi Chan, Dr. Sravanti Kusuma, Dr. Laura Dickenson, Dr. Erbil Abaci, Dr. Maureen Wanjare, Dr. Tom Yu-I Shen, Quinton Smith, Sebastian Barreto, Michael Blatchley, Ying Wang, and Daniel Lewis. I would especially like to thank Dr. Abigail Heilscher, who gave me the chance to work on her many projects, and taught me the fundamentals to be a diligent research scientist. I would also like to thank Sebastian Barreto for giving me the chance to be a part of this wonderful, and amazing project that I had the chance to work on this past year. I would also like to thank Brian Ginn from the Mao Lab for collaborating with Sebastian and I throughout this entire project.

Finally, I would like to thank Dr. Hai-Quan Mao for agreeing to be on my committee, continuously giving his opinion and sharing his knowledge on this project

and taking the time to witness the conclusion of my master's degree.

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Introduction

One of the main hurdles in tissue engineering is the need for reconstructing various types of complex structures with specific macroscopic geometries and also detailed microscopic features. Microvasculature is essential to the human body for it connects the smallest system within the blood vessel network (the capillary beds) to larger arteries and veins. Its main function is to distribute blood to various tissues throughout the body and is required for nutrient, gas, and waste diffusion between the blood and the cellular and matrix components of vascularized tissues[1]. Microvasculature is a multi-layered tissue in which there is an endothelial inner layer and an outer layer composed of vascular smooth muscle cells, and their corresponding extracellular matrix (ECM). The inner layer of endothelial cells responds to the flow regime of blood and is aligned with the direction of flow. There are many types of microvasculature, which include the commonly known arterioles, venules, as well as metarterioles — which link arterioles and capillaries[1].

The need to engineer microvasculature has increased due to a rise in vascular diseases as well as accidents that can cause a breakage of vasculature. Considering these reasons, many different approaches have been taken to begin the construction of these microvascular structures. One of the first approaches was to use microfluidic devices to try to mimic the shear stress conditions within the vessels [2-5]. However, a drawback of these systems is that their cross section is rectangular, not fully mimicking the vasculature in the body. Other techniques used to create microvasculature are three-dimensional (3D) printing, where cells are directly deposited on substrates such as collagen gels to copy three dimensional microvasculature[6]. Another technique used is

to create the structures with the help of biomaterials such as collagen gels, a process that involves the use of polymers to create a basis for which cells can adhere and grow within and later degrade naturally in the body[7-9].

Once these approaches to the construction are stable, the structures created can then be used for various applications. A common use is for drug testing and delivery to drug through microvasculature walls. However, none of the methods mentioned above can be used for another application that has a lot of potential; microsurgical implantation. Tissue-engineered blood vessel surgical bypass has become popular for treatment of peripheral vascular diseases[7]. The approach used for these studies, the use of a scaffold to create microvasculature, is able to create structures that can be biocompatibility implanted without waiting for integration and angiogenesis. This use of the reconstructed microvasculature is highly looked upon because it reduces the chances of the body rejecting the final product because the main components used can be harvested from the patient's blood.

Background

Microvasculature Structure

Microvasculature is a complex multi-layered tissue that consists of various different proteins and cell types, each of which play a significant role in the mechanical behavior of the structure[10]. Each individual vessel is composed of three layers; the intima, the media, and the adventitia. The intima is the innermost layer of microvascular tissue and is constantly in contact with the bloodstream and provides a critical barrier to platelet activation. Endothelial cells comprise the first section of the layer, which lay on a thin basal lamina, and a subendothelial layer made of ECM proteins, mainly fibronectin and type IV collagen [11]. The media layer is the thickest of the three and is made up of many stratus of smooth muscle cells within a matrix of various extracellular proteins, such as elastin and collagen I and III [12]. The outermost layer of microvasculature, the adventitia, is made up of collagen I and fibroblasts which are randomly arranged throughout the layer[12]. However, adventitia is usually only present in the larger class vessels.

The collagen and elastin present throughout the microvasculature are essential to the tissue because they provide tensile support, prevent ruptures, and confer elasticity to the vessel, allowing the ability to recover from potential ruptures or deformations[13]. The elastin's elastic nature across the span of the tissue controls the low strain mechanical response of the vessel to the flow regime of blood and also prevents energy from being dissipated as heat.

Fluid shear stress is also a major influence in the structure of microvasculature, for it helps create or destroy the formation of the tissue[8, 14]. For example, when microvasculature is exposed to laminar shear stress, there is a promotion of endothelial

cell quiescence, as well as an alteration of gene expression, a change in thrombotic potential, a promotion of anti-inflammatory phenotype, and an induction of flow alignment of the endothelial cells in the intima. However, when the intima is exposed to turbulent flow, the opposite occurs due to the harsh nature of the fluid at that state[8]. In many vessels, the shear stress contributes significantly to the vascular tone and structural adaptation through the endothelial cell dependent mediators[15]. Additionally, the use of fluid shear stress is used to help align certain ECM proteins such as elastin.

For the intent of this series of experiments, we desire to obtain microvasculature with an inner diameter of about 250 μm and a wall thickness of approximately 30 μm . In order to develop microvascular structures with the correct cellular and ECM composition and organization, we utilize endothelial progenitor cells (EPCs) and vascular smooth muscle cells (vSMCs). EPCs, isolated in either adult peripheral or umbilical cord blood, originate from the bone marrow and circulate throughout the blood stream. These cells are advantageous to use for angiogenic therapies or as biomarkers to assess cardiovascular disease progression because they have the ability to proliferate and generate functional offspring[16, 17]. ECFCs, a subtype of EPCs derived from umbilical cord blood, are used as the basis for the intima instead of other endothelial cells because of their many robust vasculogenic properties and proliferative potential[16].

Microfibers

Fibrinogen is a glycoprotein that is 340 kDa and is synthesized in the liver[10]. It is also found freely circulating in the bloodstream because it is one of the main components of blood clots. This creates a large abundance of fibrin in the body, making it easy to cultivate and use for patient specificity. During coagulation, fibrinogen is cleaved

by thrombin to form fibrin. Fibrin then forms lateral protofibril association, which results in the formation of a loosely assembled clot. This clot can then be used as an initial scaffold for tissue regeneration, serving as a platform for cell migration and proliferation. Fibrinogen has the ability to be an ideal tissue engineering scaffold, not only for its clotting properties, but also because it has the ability to bind with a wide array of molecules that could be beneficial from a vascular prosthetic engineering standpoint, such as vascular endothelial growth factor (VEGF).

One way to create fibrin based scaffolds is through the process of electrospinning. The procedure of electrospinning is attractive to many research projects because it is a simple process that requires very little equipment but can provide a lot of end material and can be performed either in a laboratory or in scaled up production[10]. The process begins with a polymer solution drawn through a syringe needle and charged with an electric potential in varying kilovolts. Once the solution reaches a critical electric potential, the solution jet is then collected at a target thrombin bath, which is placed a set distance away from the polymer solution, allowing for the crosslinking to occur. This work has been previously done to create aligned hydrogel microfibers from various natural polymers[18]. Electrospinning scaffolds is a good option for tissue engineering because the end product can be manipulated into a wide variety of shapes and can consist of fibers in an assortment of orientations. For the intent of these experiments we have chosen both a solid microfiber with an aligned internal and external nanotopography and a hollow conduit microfiber.

Degradation by Plasmin

Another important parameter to consider when using scaffolds created from biomaterials is degradation. More importantly, it is vital to determine if the scaffold is able to degrade in the body while leaving behind a freestanding tissue that can then adapt to the patient. Fibrin is degradable through a reaction with plasmin, an active version of the protein plasminogen[19]. The degradation process occurs when plasminogen is activated by tissue-type plasminogen activator or urokinase-type plasminogen activator[19-21]. The plasminogen is then cleaved to plasmin and then interacts with fibrin, thus degrading the fibrin into soluble fibrin degradation products regulated by α -antiplasmin.

It has been shown in many studies that vSMCs naturally produce plasminogen activators[19, 22]. This natural production allows for the cleavage of the plasmin to rapidly occur, and then the reaction between plasmin and fibrin ensues, leaving degradation products.

Goals and Aim of Thesis

The main goal of this series of experiments was to create free standing perfusable microvasculature. Previously our group was able to recreate multicellular microvascular tissue from ECFCs and vSMCs used. We used this to ensure the structural development is in the correct order. Because fibrin has the ability to degrade, the first aim was to fully degrade the fibrin scaffold and to obtain a luminal structure with a cell wall of appropriate thickness. The final aim was to create and develop a device that would help construct these robust structures. Once these microvascular structures were created and

fully matured, the next aim was to be able to perfuse the microvasculature to finalize structure development.

Experimental Methods

Cell Culture

Endothelial Colony Forming Cells (Lonza, Walkersville, MD) between passages six and nine were used for experiments and expanded in flasks coated with type I collagen (BD Biosciences, Franklin Lakes, NJ). The ECFCs were cultured in Endothelial Basal Medium 2 (EBM-2; Lonza) supplemented with EGM-2 Bulletkit (Lonza) and 10% fetal bovine serum (FBS; hyclone, Logan, UT) every other day. Once confluent, usually between three to five days, the cells were passaged with 0.05% trypsin/0.1% ethylenediaminetetraacetic acid (EDTA; Invitrogen, Carlsbad, CA).

Human vascular smooth muscle cells (ATCC, Manassas, VA) between passages five and nine were used for experiments. The vSMCs were cultured and fed with F-12K medium (ATCC) supplemented with 0.01 mg/ml insulin (Akron Biotech, Boca Raton, FL), 10% fetal bovine serum (FBS; hyclone, Logan, UT), 0.05 mg/ml ascorbic acid, 0.01 mg/ml transferrin, 10 ng/ml sodium selenate, 0.03 mg/ml endothelial cell growth supplement, 10 mM HEPES, and 10 mM TES (all from Sigma-Aldrich, St. Louis, MO).

Fabrication of Three Dimensional Fibrin Hydrogel Microfibers

Fabrication of the microfibers followed a similar protocol as described before, and were developed in collaboration with the Mao lab in the Material Science and Engineering Department at Johns Hopkins University [18]. There were two different types of microfibers used; fibrin and alginate microfibers and fibrin only microfibers.¹ To

¹ All hollow fibers were fibrin only fibers.

create the microfibers, a 2 wt% fibrinogen solution is fed through a syringe at a feeding rate of 2 ml/h and in-line mixed with or without a 1.5 wt% alginate solution at 1 ml/h. The solution is electrospun by applying a 5 kV electric potential between the needle and the collecting plate. Resulting electrospun nanofibers are then collected in a rotating bath of 50 mM CaCl_2 solution with 5 units/ml thrombin for twenty minutes to crosslink the fibrinogen, creating a ring-like sheet comprised of nanofibers. Once removed from the thrombin bath, the nanofibers are then soaked in 0.2 M sodium citrate overnight, transferred to water for 30 minutes, bundled into a microfiber, stretched, and air dried.

Fabrication of Hollow Microfibers

To fabricate hollow microfibers, the same method described above was used to create microfiber sheets by rastering the landing position of the fibrinogen jet on the collection solution. Then these microfiber sheets are wrapped around a mandrel that has been coated with PTFE (Teflon; Applied Plastics Co., Norwood, MA). From there, the hollow fibers are soaked in a very high concentration thrombin bath (100 U/ml) to further crosslink between the wrap layers create a continuous structure. Circumferential or longitudinal alignment of the fibrin structure is generated based on the orientation of the mandrel during wrap relative to the fiber sheet alignment. Once both the coiled coil and longitudinal structures are crosslinked, they are frozen and lyophilized, following which they are removed from the mandrels.

Prior to seeding, the fibers are sterilized in 75% ethanol and rinsed twice with water.

Cell Seeding and Culture on Fibrin Hydrogel Microfibers

Cell seeding on fibrin hydrogel microfibers follows the same procedure as described before[23]. Once ECFCs were confluent, they were passed and seeded on fibrin

hydrogel microfibers wrapped around custom made frames. Cells were passaged with 0.05% trypsin/0.1% ethylenediaminetetraacetic acid (EDTA; Invitrogen, Carlsbad, CA) and then seeded to the microfibers at a cell density of 2×10^6 cells in 4 to 5 ml of ECFC media supplemented with 50 ng/ml VEGF (Pierce, Rockford, IL) in custom made tubes. The tubes were then placed on a tumbler for twenty-four hours at 37°C to allow cells to attach to the microfiber. Frames were then transferred to 35 mm dishes and cultured in ECFC media supplemented with VEGF for another four days with media changes every other day. On day five of the experiment, vSMCs were seeded onto the ECFC seeded microfibers in the same way; cells were seeded at a cell density of 2×10^6 cells in 4 to 5 ml of regular ECFC media (no VEGF supplement) for another five days. Samples received a media change every other day. Ten days after the original ECFC seeding, samples were fixed and stained as described below.

Cannulating of Conduit Fibers and Perfusion

Conduit shaped fibers were cannulated using luer lock needles gauges 25 to 30. Once the fibers were secured with either surgical glue or sutures, diluted blue dye or media was directly perfused through the needles and fibers using a three port luer lock, where one port was attached to the needle, one was closed, and the third was attached to a syringe.

Three Chamber Device Set Up, Cell Seeding and Culture on String Fibrin Microfibers

Before setting up the device, all components, a glass slide cover, two screws and nuts, the top and bottom plate made of poly(methyl methacrylate) (PMMA), and six luer lock caps, were autoclaved to ensure sterility; significant pieces are shown in Figure 1[24]. At the same time, two polydimethylsiloxane (PDMS) blocks were cut to the size

25 mm x 10 mm, with a slit cut 3 mm from the bottom of each block and polyester shrink tubing with an inner diameter of 250 μ m was cut into 10, 8 mm long pieces. Five pieces of tubing was then placed parallel to one another throughout the slit at the bottom of each block with 2 to 3 mm left on either side. Once the tubing was fed through, the sides facing each other had that tubing coated in type I collagen for one hour. Once the allotted time was finished, five fibrin microfibers were then fed through the tubing of both blocks.

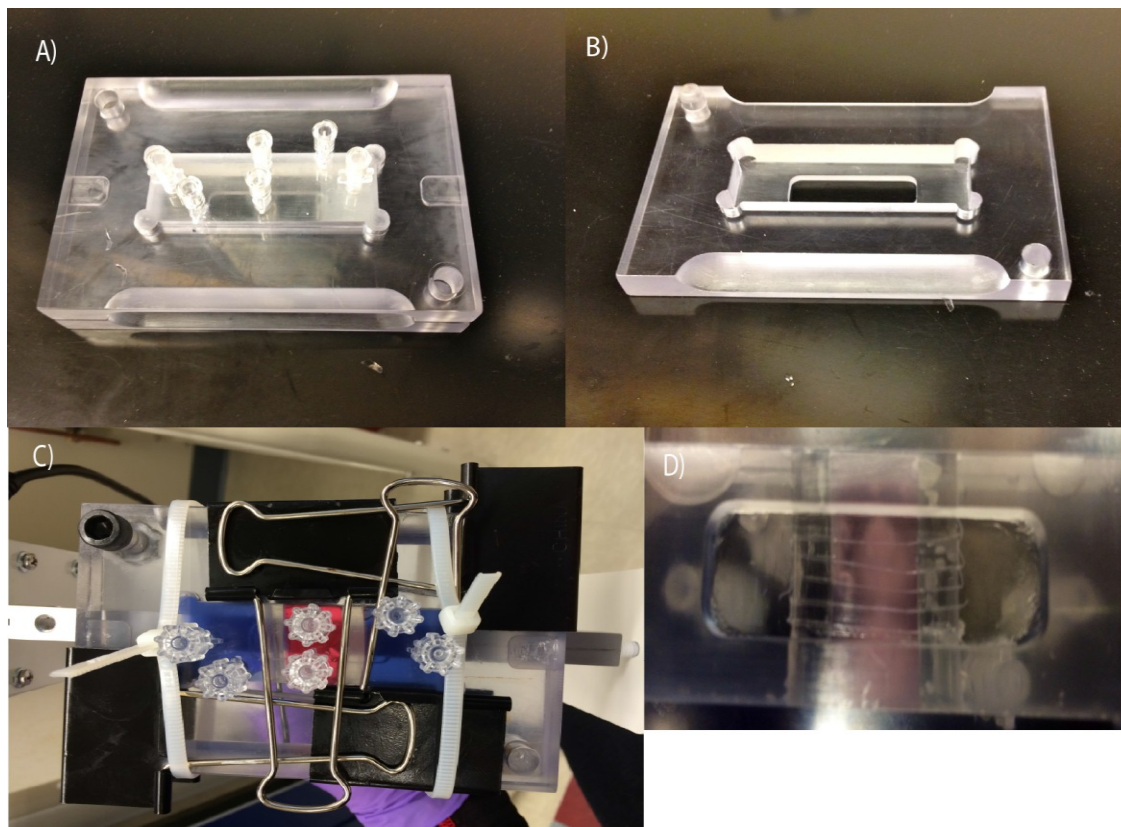


Figure 1: Three Chamber Device Set Up showing the (A) top and (B) bottom plates separately. Three chamber device set up after a cell seeding is shown in (C). The device consisted of two plates placed onto each other with a small chamber cut out in the center of each plate. The plates were secured with vacuum grease to ensure a seal. Additionally, two nuts and bolts were used to keep the plates in place and four binder clips were placed on each side of the device to make sure the plates stayed together. (D) The bottom of the three chamber device shows how the PDMS blocks create the three distinct chambers.

To assemble the device, all components along with the PDMS – fiber bridge were brought into the biological safety cabinet. Vacuum grease was applied to the interface of the two plates, leaving a 1 cm gap from the inner chamber of the device on both plates.

Additionally, vacuum grease was applied to the bottom of the glass cover slip and then the side that was coated was placed in the inner compartment of the bottom plate and pressed firmly around the edges to confirm a seal. Once everything has been coated with vacuum grease, all parts including the PDMS – fiber bridge were then UVed for 30-45 minutes. The blocks with the fibers were then placed on the center of the glass cover slip. The fibers were then pulled taut and trimmed to ensure the smallest amount of fiber and tubing combination was in the outer compartments. Then, sterile water was placed in the center compartment to help hydrate the fibers, ensuring they swell in the tubing and remain taut and stable throughout the length of the experiment. The device was sealed by pressing the two plates together firmly, screwing in the screws, and sterilizing with 75% ethanol and rinsed with sterile water three times via luer lock ports. Leaving some water in the middle compartment to prevent microfiber dehydration, the device was then placed in a 37°C incubator overnight to ensure a vacuum seal. The next morning, the exterior compartments are filled with phosphate buffered saline (PBS) throughout the duration of the experiment.

Cell seeding on fibrin fibers followed the protocol described above. ECFCs were seeded to the middle compartment of the device at a cell density of 2×10^6 cells in 3 ml of ECFC media supplemented with 50 ng/ml of VEGF. The device was then secured to a tumbler for twenty-four hours at 37°C to facilitate cell adhesion. Media was changed every other day for five days. On day five of the experiment, vSMCs were then seeded to the center compartment that contained the ECFC seeded microfibers at a cell density of 2×10^6 cells/ml with ECFC media. The device was once again attached to the tumbler at

37°C for another twenty-four hours with media changed every other day for another ten to fifteen days. Overview of the entire experiment is shown in Figure 2.

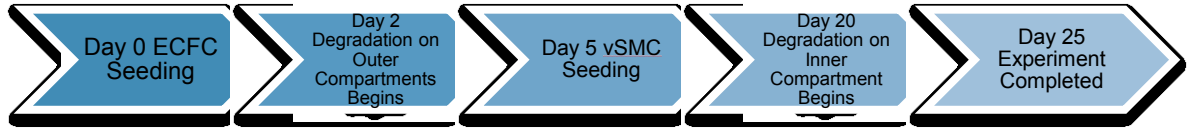


Figure 2: Time Frame for Cell Seeding for Microvasculature Reconstruction

Single-Chamber Device Set Up, Cell Seeding and Culture on Conduit Fibrin Microfibers

Before setting up, borosilicate tubing (Friedrich and Dimmock Glass, Millville, NJ) with dimensions 13 mm x 26 mm cut in 38 mm pieces were sonicated and then autoclaved to ensure sterility. Simultaneously two PDMS blocks were cut to 15.5 mm x 24 mm. A one inch 25 gauge blunt tip luer lock needle was then punctured 3 mm from the bottom of both PDMS blocks and at the top left corner of each block, a size 14 gauge blunt tip luer lock needle was punctured through both blocks as well. The conduit fibers were then cannulated and sutured between the two 25 gauge needles as show in Figure 3. The set up was then moved to the biological safety cabinet, where ethanol was added to fill the entire chamber using the size 14 gauge needle at the top corner. Once the chamber was filled, the system was further sterilized by UV treatment for 30 minutes. Afterwards, the chamber was washed with sterile distilled water three times via the top luer lock needle. The bottom needles were locked until the end of the experiment when perfusion takes place.

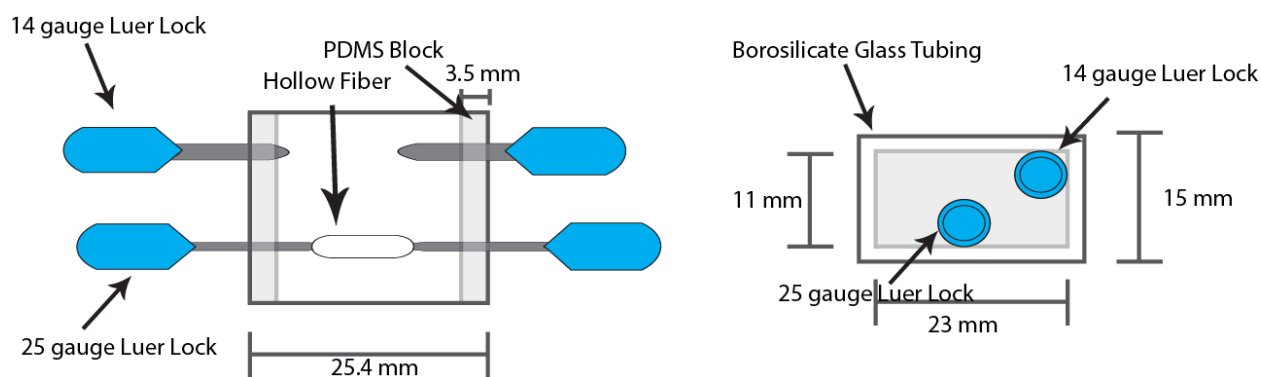


Figure 3: Design of Single Chamber Device (not drawn to scale)

Cell seeding on conduit fibers followed same protocol described for the three chamber device.

Degradation of Fibrin Microfibers

Microfibers with or without cells were treated with plasmin at 15, 1, 0.25, and 0.1 CU/ml (Athens Research and Technology, Athens, GA) in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies, Carlsbad, CA) for 1, 6, 12, and 24 hours, respectively, and monitored regularly. Conduit fibers without any cells attached were treated with 0.25 CU/ml plasmin and DMEM for 12 hours.

Plasmin Treatment in Three Chamber Device

At day two of the experiment using the three chamber device, the outer compartments were treated with a degradation media consisting of 9 $\mu\text{g/ml}$ of plasmin (equivalent to about 0.25 CU/ml), in DMEM that was changed every five days. At day twenty the inner compartment was treated with the same degradation media as the outer compartments, and media was refreshed every 12 hours.

Immunofluorescence Staining and Confocal Microscopy

Cell-microfiber samples were fixed with 3.7% formaldehyde (Fisher Chemicals, Fairlawn, NJ) for fifteen minutes, permeabilized with 0.1% Triton X-100 (Sigma

Aldrich) solution for ten minutes, and then blocked with 0.01% bovine serum albumin (Sigma Aldrich) for one hour. After washing the sample three times with PBS, primary antibodies were then added to the samples and incubated for one hour at the dilutions shown in Table 1. After another three washes of PBS, samples were incubated with secondary antibodies, Alexa Fluor 546, and Alexa Fluor 647 (Invitrogen), or conjugated phalloidin (Invitrogen) at room temperature for one hour. Samples were washed with PBS another three times and then counterstained with DAPI for ten minutes. Z-stack images were taken using confocal microscopy (LSM 780 Carl Zeiss Inc., Thornwood, NY) in order to further analyze samples.

Reagent Type	Name	Host	Dilution Used
<i>Primary Antibodies</i>	Type IV Collagen	Mouse/Rabbit	1:100
	Fibronectin	Rabbit	1:100
	Elastin	Mouse	1:100
	VECad	Mouse	1:100
	SM22	Rabbit	1:100
<i>Secondary Antibody</i>	Alexa 546	Donkey	1:500
	Alexa 647	Donkey	1:500
<i>Conjugated Antibody</i>	Phalloidin 488	Shroom	1:50
	DAPI	N/A	1:1000

Table 1: Immunofluorescence Dilutions used during staining of microvasculature samples constructed.

Results

Development of Microvascular Structures on Frames

Previously our group devised a protocol to develop microvasculature using fibrin microfibers [23]. We seeded ECFCs onto microfibers wrapped around frames for five days followed by vSMCs for another five days. These samples were then fixed and stained for various ECM proteins to show their organization and ensure cell alignment along the string-like microfiber. Microfibers wrapped around frames were then degraded

to create multicellular structures with a lumen after the ten day culture period. However, we wanted to develop a more robust smooth muscle cellular layer that would withstand perfusion. To do this we extended the vSMC culture time from five days to ten to fifteen days.

Degradation Studies to Remove Fibrin Core

To be able to use the microvasculature created it was necessary to begin degradation studies of the scaffolds. We begun by treating the fibrin and alginate-fibrin microfibers with different concentrations of plasmin. We found that plasmin based degradation is concentration dependent as shown in Figure 4. We treated microfibers with concentrations of 15, 1, 0.25, and 0.1 CU/ml plasmin, which degraded the microfibers in 1, 3, 6, 12, and 24 hours respectively. From the different concentrations, we determined that 0.25 CU/ml plasmin treatment for 12 hours and the 0.1 CU/ml plasmin treatment for 24 hours were the most suitable for the experiments to follow. The 12 and 24 hour treatments were also performed on microfibers made from fibrin only (no alginate) as shown in Figure 5.

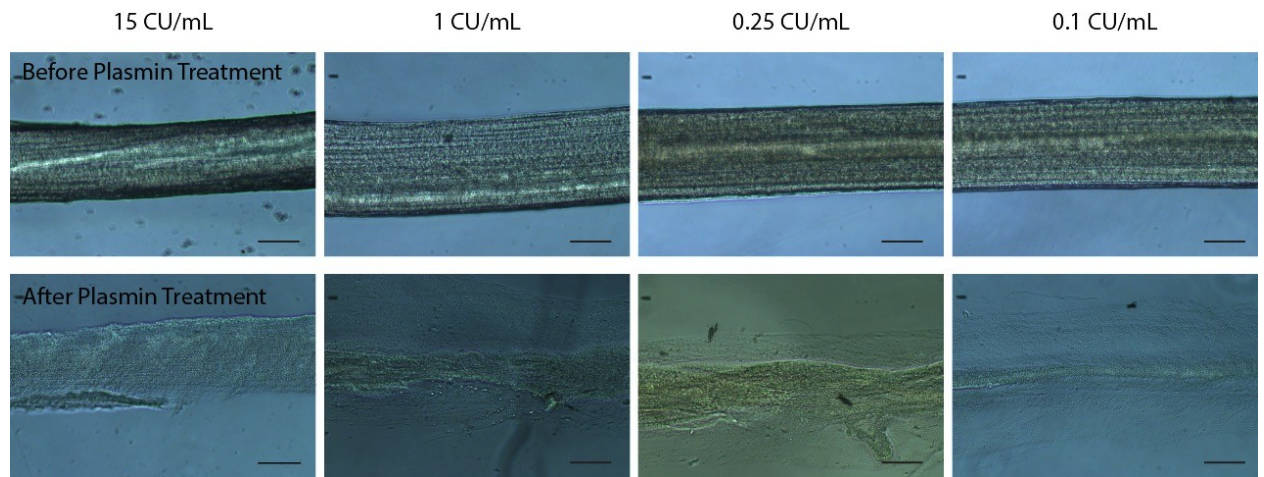


Figure 4: Fibrin-Alginate hydrogel microfiber degradation using a plasmin treatment with various concentrations of 15, 1, 0.25, and 0.1 CU/mL plasmin for 1, 6, 12, and 24 hours respectively. Scale bar = 100 μ m.

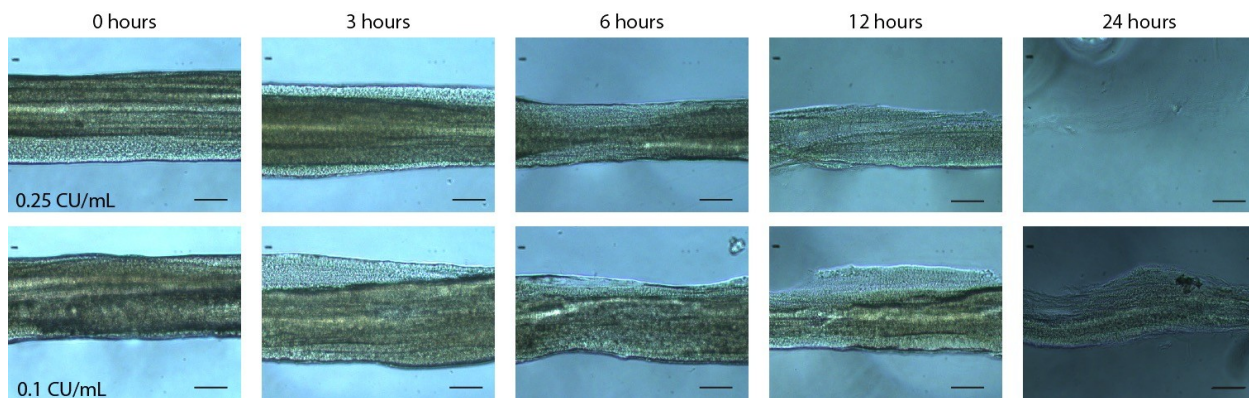
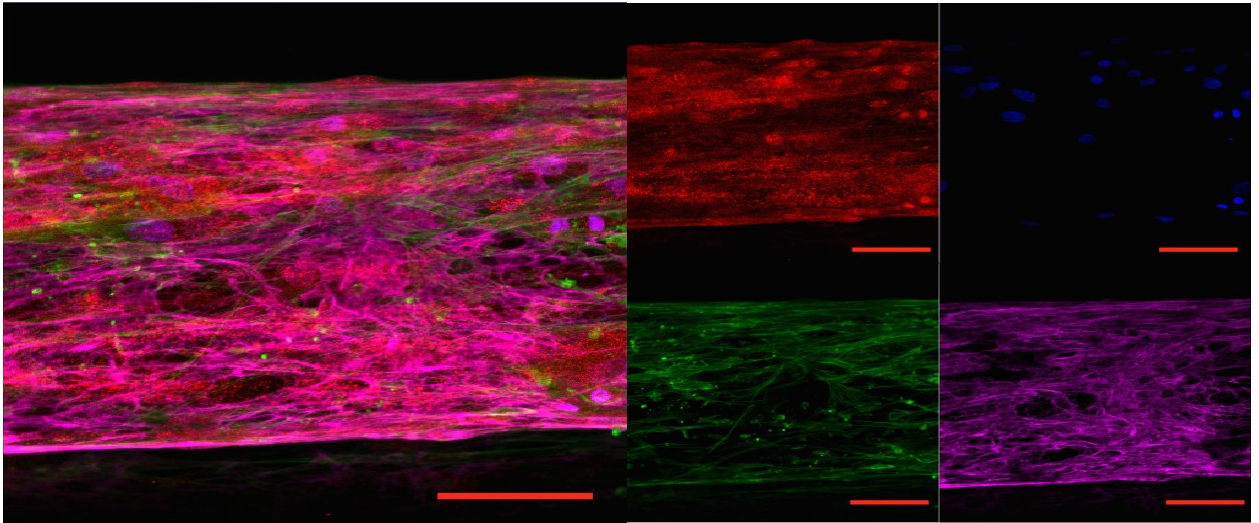


Figure 5: Fibrin only hydrogel microfiber plasmin degradation with plasmin concentrations of 0.25 and 0.1 CU/mL for 12 and 24 hours respectively. Scale bar = 100 μ m.

Once it was established that degradation of the scaffold was possible, we conducted experiments to see how the plasmin degradation media would affect the cell layers cultured onto the microfibers. We seeded ECFCs onto microfibers wrapped around frames for five days followed by vSMCs for another five days. After the ten days, we fixed, stained, and imaged samples and then administered a 0.25 CU/ml plasmin treatment on the microvascular structures for 12 hours. This showed that the plasmin treatment would not affect the cell layers and keep the ECM organization intact. After treatment, we analyzed the samples for degradation of fibrin microfibers as well as lumen formation. Using confocal imaging, we found that each cell layer was still intact after the plasmin treatments as shown in Figure 6. Immunofluorescence staining of the structures revealed a large deposition of various ECM proteins such as elastin, and fibronectin. Because we could not achieve full degradation of the fibrin-alginate core, we used fibrin-only microfibers for the remainder of the study.

Before 12 Hour Degradation



After 12 Hour Degradation Treatment

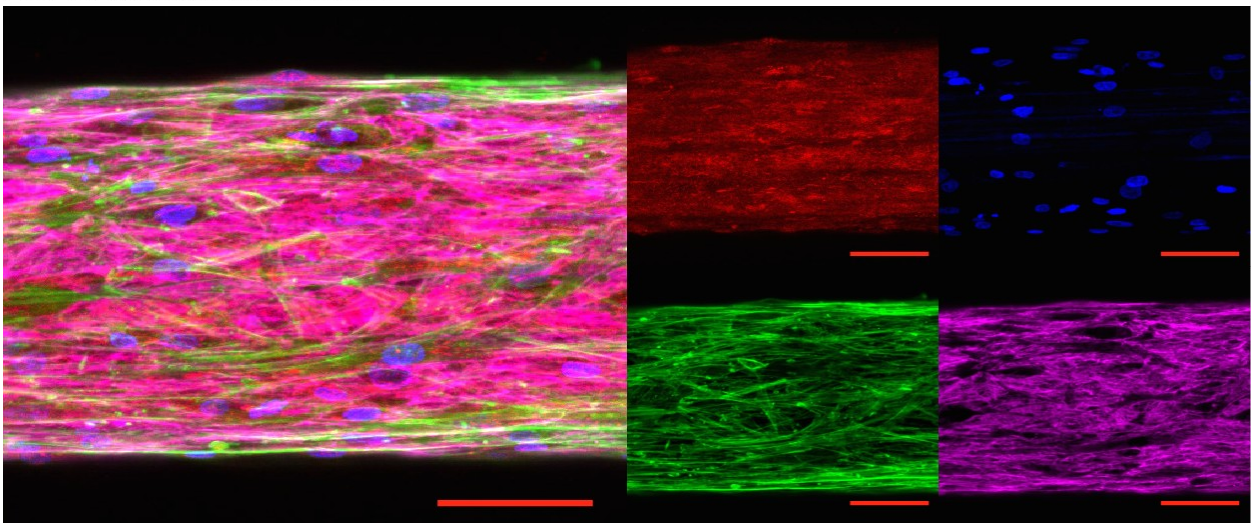


Figure 6: Initial degradation studies on frames: ECFCs were seeded and cultured onto fibrin-alginate microfibers for five days followed by vSMC which were cultured for another five days on frames. The samples were then fixed and stained for elastin (red), fibronectin (magenta), f-actin (green), and nuclei (blue). Cocultures then went through a degradation treatment of 0.25 CU/mL plasmin for 12 hours and reimaged to see if the cell layers were affected. Scale bars = 100 μ m.

Live Cell Culture on Microfiber and Degradation on Frames

After demonstrating that the cell layers would not be affected by the plasmin treatment when tested on frames, we then decided to first test the plasmin treatment on a live cell culture using the frame set up. We cultured ECFCs on fibrin only microfibers on frames for five days followed by vSMCs for another fifteen days. After cells were

cultured for twenty days, we administered 12 hour treatments of 0.25 CU/ml plasmin media to live co-cultures followed by a 12 hour recovery in regular culture media up to a total of four cycles.

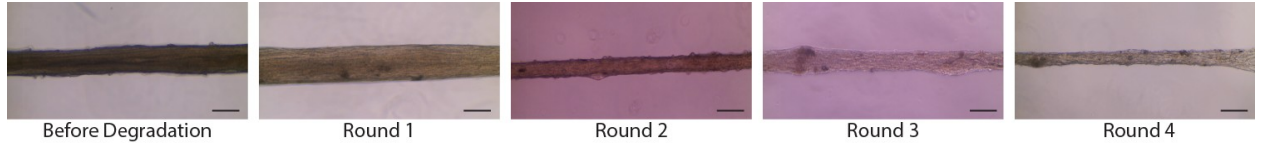


Figure 7: Brightfield images of live cell culture degradation: ECFCs were seeded onto fibrin only microfibers for five days followed by vSMCs were then cultured for another fifteen days on frames. Microvasculature cell culture on fibrin only microfibers were then given a degradation treatment for 12 hours with 0.25 CU/mL plasmin and then immediately given a twelve hour recovery period with full serum ECFC media. This was repeated four times to demonstrate that the microfiber was able to degrade while cells were attached. Scale bar = 100 μ m.

We found that after one treatment of degradation media, a portion of the microfiber had degraded indicating that fibrinolysis had occurred. Additionally, a greater amount of fibrin degraded with each administration of the plasmin treatment. Figure 7 shows the progressional degradation of the fibrin microfiber after each round of plasmin treatment with twelve-hour recovery periods in ECFC culture media. Afterwards, we fixed and stained samples for F-actin, type IV Collagen and Elastin, and then imaged using confocal microscopy to ensure ECM deposition was still in tact, as shown in Figure 8.

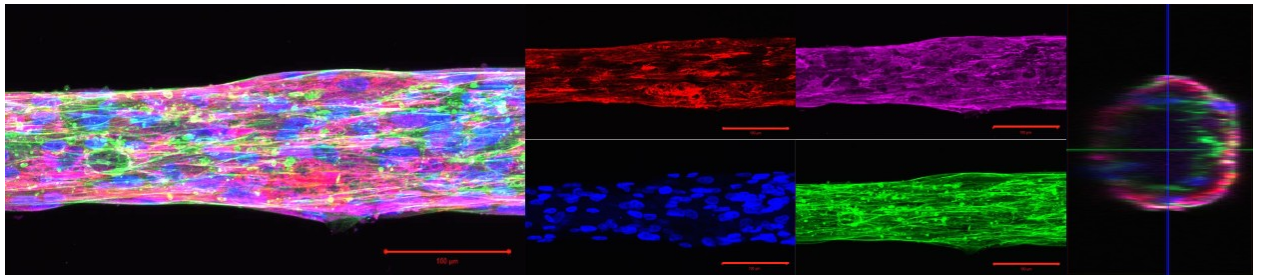


Figure 8: Microvasculature with a fully degraded fibrin core: ECFCs were seeded onto fibrin only microfibers for five days followed by vSMCs were then cultured for another fifteen days on frames. Microvasculature cell culture on fibrin only microfibers were given a degradation treatment for 12 hours with 0.25 CU/mL plasmin and then immediately given a twelve hour recovery period with culture media four times. Sample was then fixed and stained for type IV collagen (magenta), elastin (red), f-actin (green), and nuclei (blue). This demonstrated that it was possible to fully degrade the fibrin-only fiber leaving a lumen as shown above. Scale bar = 100 μ m.

The degradation of the fibrin microfiber demonstrated that it was possible to break down the scaffold and lead to partial lumen formation. However, the structure constructed also began to collapse due to SMC contraction in the absence of flow. This gave us motivation to create a device that would allow us to perfuse the developing structures immediately following fibrin core degradation.

Three Chamber Device

After the success of the previous experiments on frames, we created a device to construct freestanding microvasculature, which would then have the fibrin microfiber core degraded, allowing perfusion of fluids through the new structures. A three-chamber device was created to allow the construction of freestanding microvasculature as shown in Figure 1. The device consists of two PMMA plates placed onto each other with a small PMMA chamber cut out in the center of each plate. The plates were secured with vacuum grease to ensure a seal. Additionally, we used two nuts and bolts to keep the plates in place and then placed four binder clips on each side of the device to make sure the plates stayed together. To create the three chambers, two blocks made of polydimethylsiloxane (PDMS) were evenly separated throughout the chamber. The PDMS blocks then held up the microfibers, so that individual scaffold samples did not rely on other samples to stay taught and straight throughout the experiment.

We first seeded ECFCs onto the microfibers in the inner chamber of the device for five days. Following this, vSMCs were seeded on top of the ECFCs and then cultured for another 15 days. At day twenty of the experiment, we placed degradation media (0.25 CU/ml plasmin in DMEM) in the two outer chambers for the remaining of the experiment to allow degradation of the microfibers. After the allotted vSMC culture had elapsed, we

administered four rounds of plasmin degradation treatment to the inner compartment. After each degradation treatment, the samples had a 12 hour recovery in culture media. When we established a protocol to create the microvasculature within the inner compartment of the chamber, we still had many issues with fully degrading the fibrin scaffold within the inner compartment.

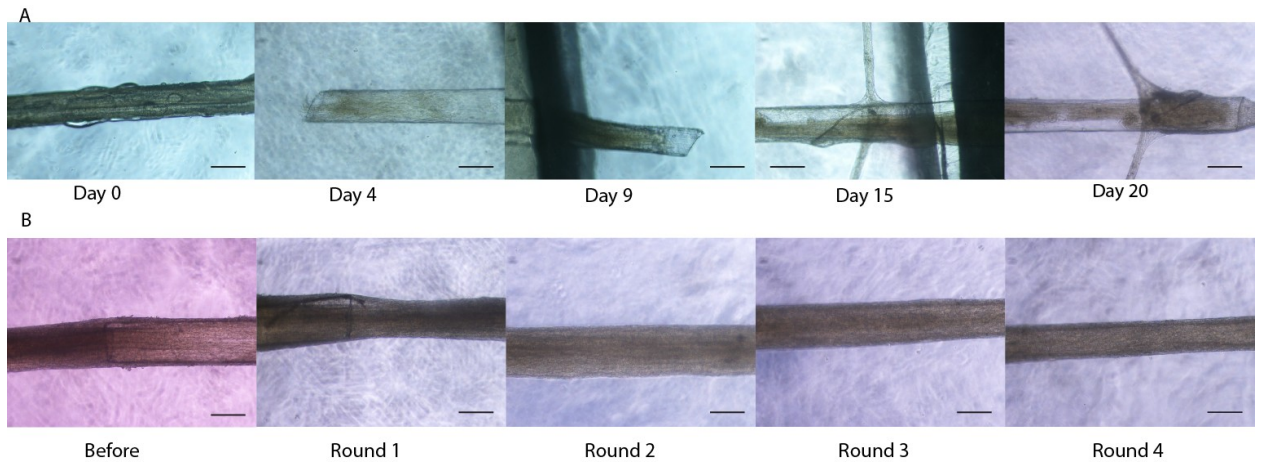


Figure 9: Progression of Fibrin Core Degradation in the Three Chamber Device: ECFCs were cultured onto fibrin only microfibers for five days followed by vSMCs for another fifteen days in the three-compartment device. Samples were administered the degradation plasmin treatment of throughout the experiment in both (A) the two outer compartments and (B) the inner compartment. The degradation process began on day 2 for the outer compartments and at day 20 for the inner compartment with 0.25 CU/ml plasmin with DMEM. For the inner compartment plasmin treatment was administered for twelve hours with a twelve-hour recovery period in full serum ECFC media; this process was repeated three times. Scale bar = 100 μ m.

To improve degradation in the device, we started degradation treatments in the two outer compartments at day two of the experiment; changing the degradation media every five to six days. These changes resulted in the microfibers degrading faster in the outer compartments, as shown in the bright field images of the progression of the degradation treatments in Figure 9. Although the amount of fibrin microfiber degraded had increased from the previous experiments, the thicker cell layer resulting from longer culture periods prevented full microfiber core degradation and subsequent lumen formation. We then fixed, stained, and imaged samples for deposition of ECM proteins F-actin, Elastin and Type IV Collagen as shown in Figure 10.

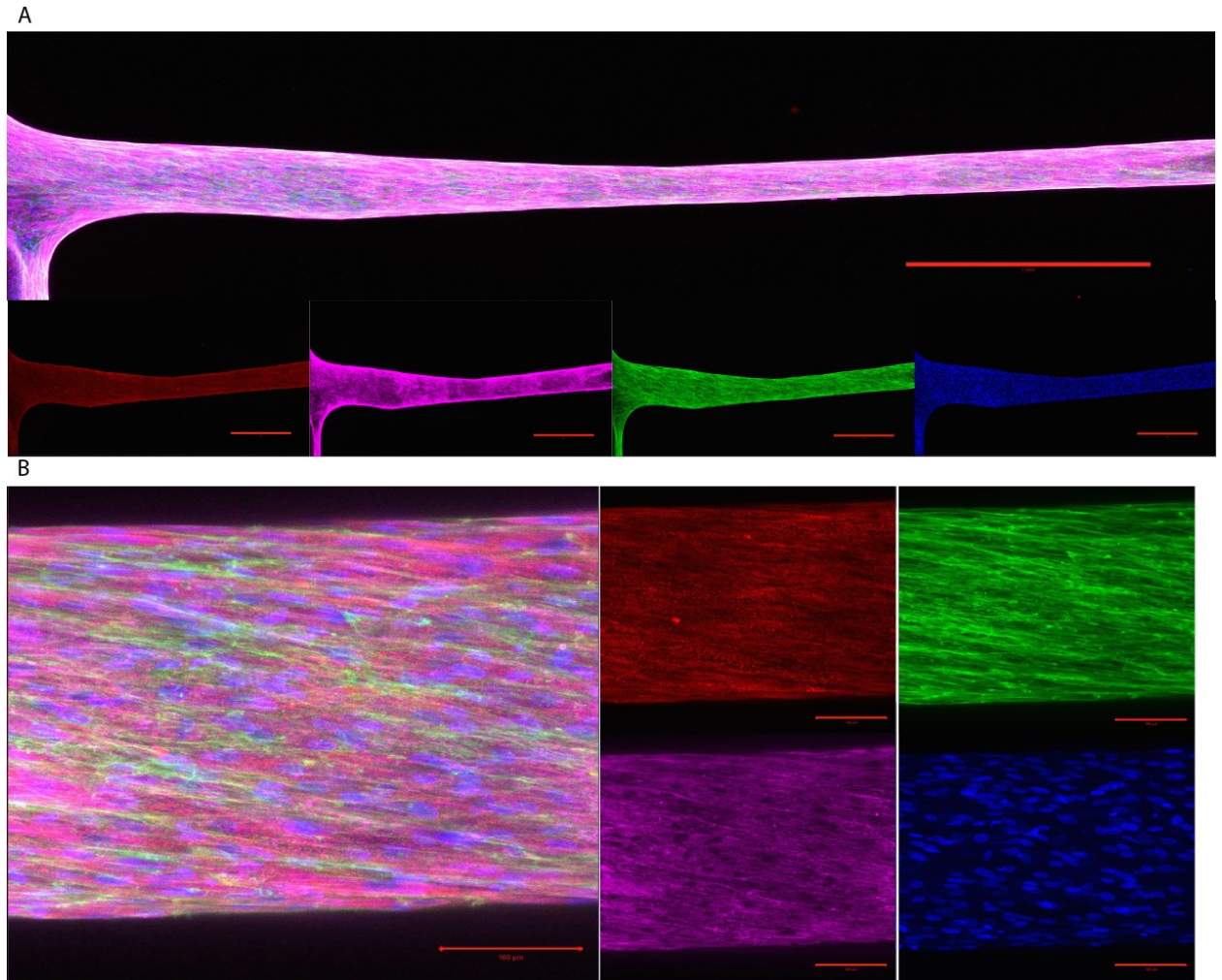


Figure 10: Microvasculature with partially degraded fibrin core: Samples in three chamber device were ECFCs cultured on fibrin only microfibers for five days followed by vSMCs cultured on the microfibers for another fifteen days. Samples were then administered four rounds of degradation media immediately followed by a twelve hour recovery period in culture media. Microvasculature was then fixed and stained for type IV collagen (magenta), elastin (red), f-actin (green), and nuclei (blue) after 20 days of cell culture, shown in (A) 10x magnification (Scale Bar = 1 mm) and (B) 20x magnification (Scale Bar = 100 μ m).

Another method that we attempted to increase the amount of fibrin microfiber degradation was to shorten the size of the microfiber used for the scaffold of the microvascular structures. This is because the smaller the amount of material used, the faster the degradation of the scaffold. Combining the two techniques proposed a way to optimize the amount of microfiber degradation in the device. However, as shown by Figure 11, this attempt was also unsuccessful, demonstrating the exact opposite of what

we hypothesized. Even less of the material was degraded than the previous two runs of the experiment. We fixed and stained the structures to show the endothelial cell and vSMC structure with vascular endothelial cadherin and vSMC marker SM22 to demonstrate how the two cell types lay upon each other on the scaffold; this is shown in Figure 12. We found that there was no vascular endothelial cadherin expression, demonstrating that the vSMCs did not reorganize the cellular layers.

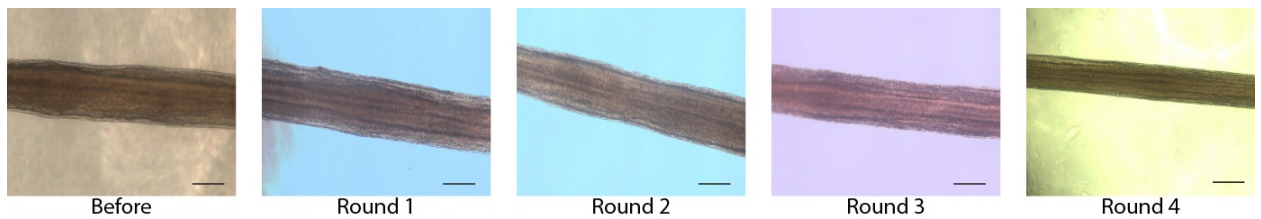


Figure 11: Second Experiment in Three Chamber Device: ECFCs were cultured onto fibrin only microfibers for five days followed by vSMCs for another twenty days in the three-compartment device. Samples were administered the degradation plasmin treatment (0.25 CU/ml plasmin in DMEM) in the inner compartment beginning at day 20. A twelve-hour recovery period in full serum ECFC media was given immediately after the twelve-hour treatment; this process was repeated three times. This run of the three chamber device used the smallest possible length of microfiber scaffold to see if full degradation was possible in the device similar to that of on the frame. Scale bar = 100 μ m.

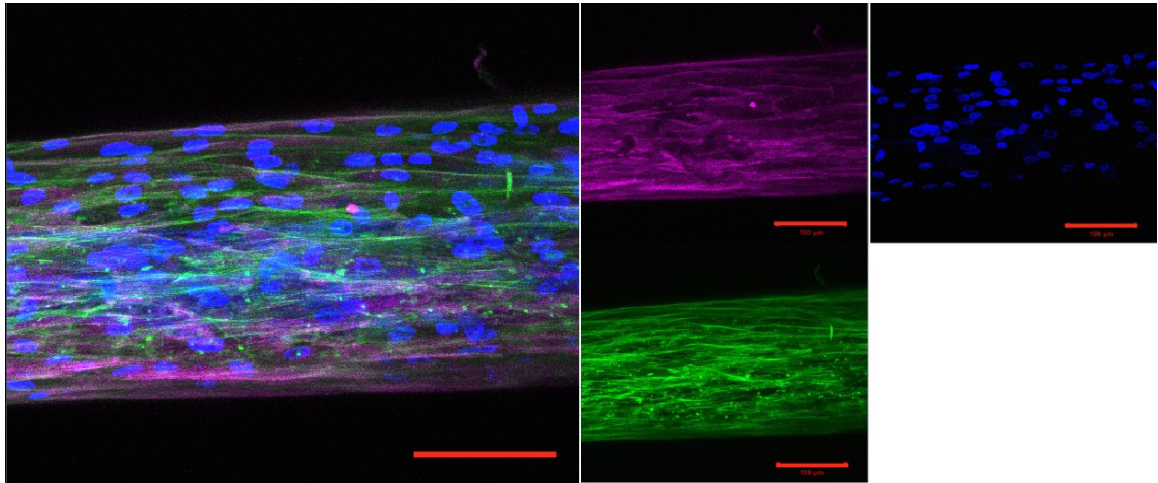


Figure 12: Endothelial Cell and Smooth Muscle Cell positioning of microvasculature: Samples in three chamber device were ECFCs cultured on fibrin only microfibers for five days followed by vSMCs cultured on the microfibers for another twenty days. Samples were then administered four rounds of degradation media immediately followed by a twelve hour recovery period in full serum ECFC media. Microvasculature created were then fixed and stained for SM22 (magenta), f-actin (green), and nuclei (blue) after 25 days of cell culture. This demonstrated that the SMCs and ECFCs did not move around, but stayed in the three layers of microvasculature. Scale bar = 100 μ m.

We were unable to obtain full degradation of the fibrin only microfibers in this set up due to plasmin diffusion through the thick cell layer, as well as fibrinolysis products being stuck inside the lumen that was created. Based on the three unsuccessful attempts, we designed a new system and microfiber shape to be one step closer to the main goal of creating perfusable freestanding microvascular structures.

Introduction of Conduit Hollow Fibers

While troubleshooting the three chamber device, the first component that we changed was to alter from cylindrical microfibers to hollow conduit microfibers. We fabricated the hollow microfibers of the same material as the microfibers; fibrin. The main difference between the two is that there is a lumen in which fibrinolysis products can leave.

First, we treated the hollow microfiber samples without cells with 0.25 CU/ml plasmin degradation media for 24 hours as shown in Figure 13. This was to demonstrate that the hollow microfibers were able to fully degrade similarly to regular microfibers.

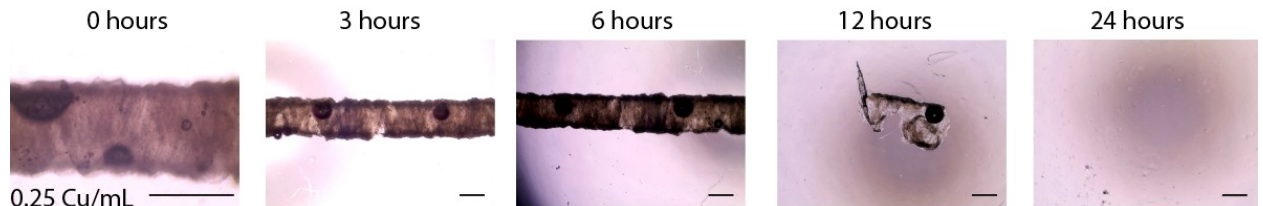


Figure 13: Coiled fibrin only hydrogel microfiber degradation using a 0.25 CU/mL plasmin treatment for 12 hours. Scale bar = 500 μ m.

The most significant aspect of this particular shape was to ensure that there was no leaking while perfusing through the hollow fibers. We implemented three different methods of attaching the hollow microfibers to blunt needles for perfusion: keeping the hollow fibers un-tethered, using surgical glue to fix them to the needles, and finally suturing the ends of the hollow fiber to the needles, anchoring them in a stable position.

We found that leaving the hollow microfibers un-tethered resulted in fibers falling off or leakiness at the ends of the fiber where it was attached to the needle. We also found the microfibers were absorbent to surgical glue, making its use undesirable. Meanwhile, sutures allowed the ends of the microfibers to be well anchored on the needles so they would not fall off during the twenty four hour tumble period of each cell seeding. Furthermore, there was minimal leaking at the ends when perfusing the microfibers without cells. After many trials we determined that suturing the microfibers was the best option.

One Chamber Device

When it was established that the new hollow fibers would not move from the needle, we created a single chamber device based on the design of the three chamber device. We utilized one inch pieces of borosilicate tubing as the chamber, and PDMS blocks capped the ends of the tubing. Each block holds two luer locks, one for media changes and the other for hollow microfiber perfusion. The hollow fiber is connected to the luer lock needles on both sides and secured with sutures to ensure that it will not fall off the needles throughout the experiment. The design of this device is shown in Figure 14.

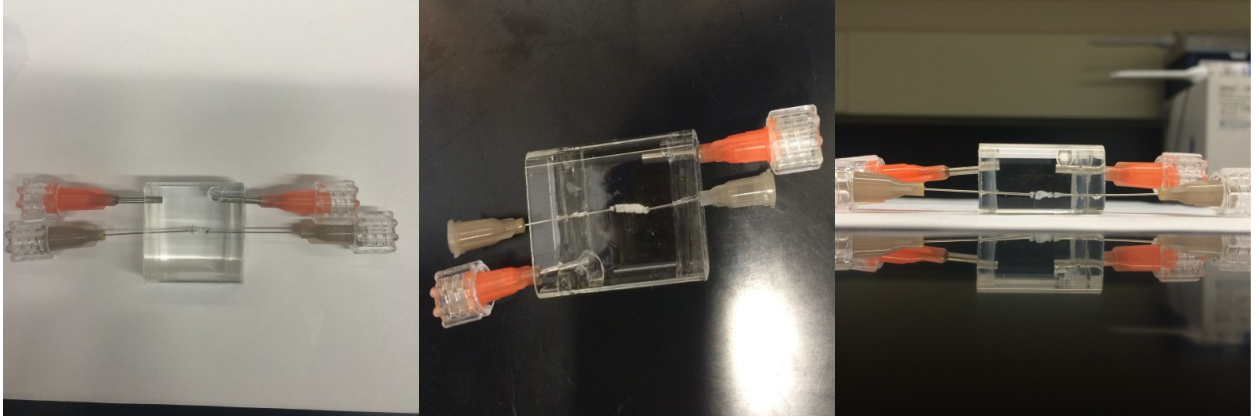


Figure 14: Single chamber device set up with conduit coil style hollow fiber sutured to luer lock needles.

We then conducted a trial experiment with ECFCs for five days to see if having a cell layer would prevent leaking. We then fixed and stained the samples for F-actin, type IV Collagen, and Fibronectin to determine if the cell or ECM organization had changed at all due to the change of topography of the coil like hollow fiber, as shown in Figure 15. After confocal imaging, the structure demonstrated that the coil topography slightly changed the cell alignment of the ECFCs compared to that of the microfibers, which had previously shown to follow the longitudinal topography of the microfibers. We then perfused the structure with a fluorescent dye and demonstrated that a more robust cell layer was needed before perfusion could take place.

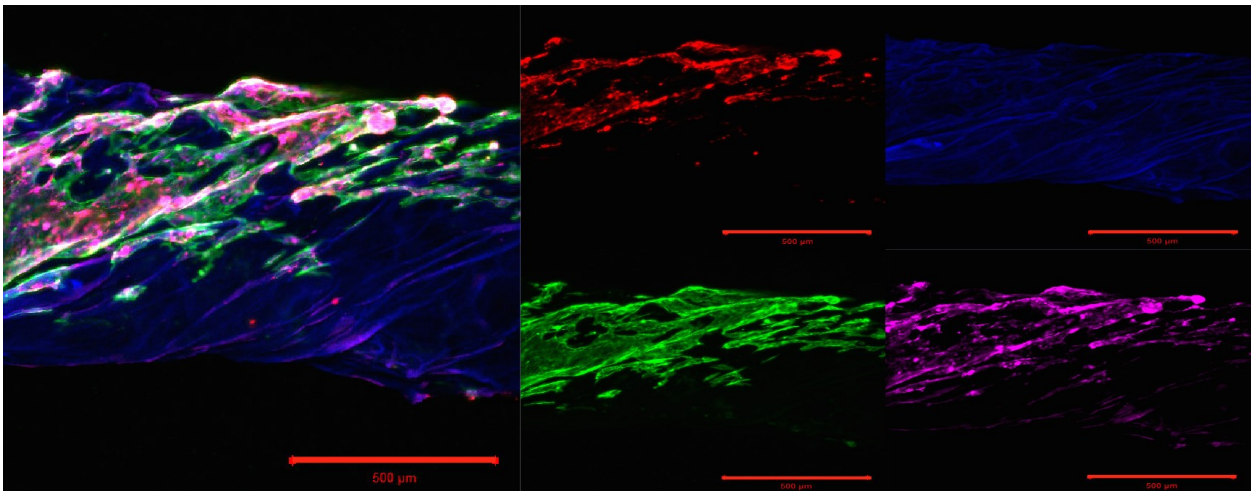


Figure 15: Initial experiment with coiled style hollow fiber with ECFCs seeded on them for five days in single compartment device. Samples were then fixed and stained for type IV collagen (red), fibronectin

(magenta), and f-actin (green). The blue stain is to show the topography of the microfiber, demonstrating that the cells adhere to the ridges of the coils, instead of aligning along the fiber itself. Hollow microfibers are also twice as large as the microfibers. Scale Bar = 500 μm .

We conducted another set of experiments with ECFCs cultured on the hollow fibers for five days. We also fixed and stained the structures for F-actin, Type IV Collagen and Fibronectin to see if the topography was similar to that in Figure 16. As shown in Figure 16, we found that the ECM was able to align perpendicular to the longitudinal axis of the microfiber and the alignment cues of the microfibers took precedence instead of the ridges in the topography of the microfiber. After imaging, we perfused the structure with DMEM to ensure minimal leakage from the structure and found that the microvasculature we created was able to perfuse media with a small amount leaking through.

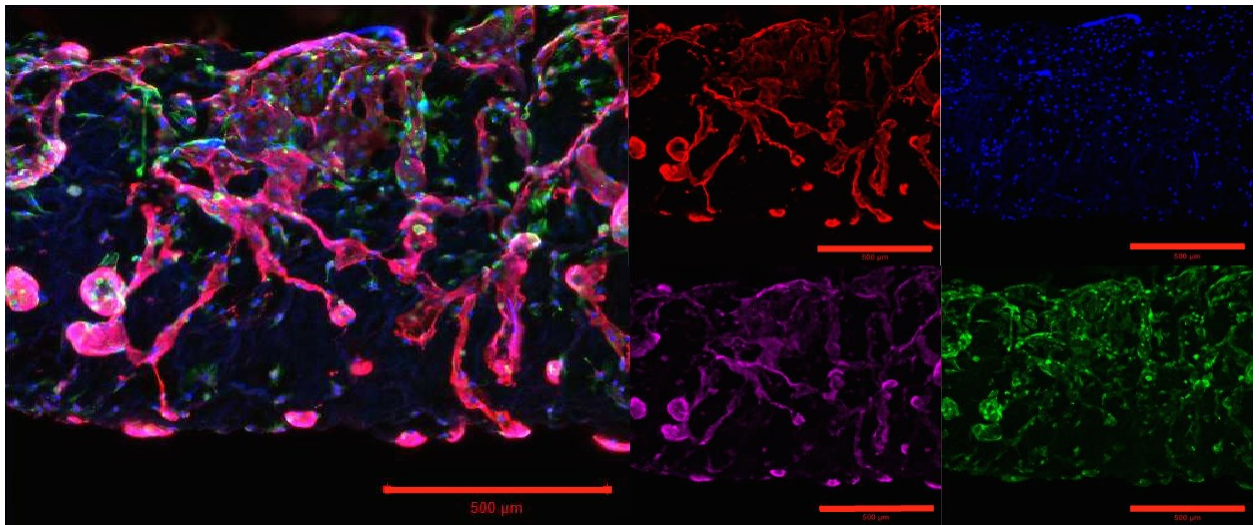


Figure 16: Second experiment of hollow fibers with ECFCs cultured for five days in single compartment device. Samples were then fixed and stained for type IV collagen (red), fibronectin (magenta), and f-actin (green). Scale Bar = 500 μm .

Discussion

The overall goal of this study was to create free standing perfusable microvascular structures from a fibrin microfiber scaffold. The first significant step towards this aim

was to create structures that mimicked each layer of microvasculature. Previously our group had shown that it was possible to create a scaffold that would incorporate alignment topography. This would ultimately help cellular organization, as well as how both the endothelial cells and vascular smooth muscle cells used would adhere and align to the fibrin scaffold and produce an optimal ECM deposition[18, 23, 25]. Furthermore, this system was shown to be able to recapitulate the wrapping ECM deposition surrounding the endothelium, as seen in native blood vessels. Additionally our group demonstrated that it was possible to create a multicellular microvascular structure with a lumen, however the culture time for these structures was only ten days total[25]. Additionally, these results were based on the microfibers that were held up by frames to maintain a taut, straight, foundation for the cells. Due to the success of these experiments we proceeded to construct a more robust tunica media, degrade the microfiber core, and continue maturation by applying flow.

To degrade the fibrin microfiber scaffold, it was pertinent that the proper concentration of plasmin and time was used to find the optimal conditions that would allow the fibrin to go through fibrinolysis without killing too many of the cells. We chose the 12 hour treatment as the best choice because it allowed a long timeframe for the plasmin to interact with the fibrin without creating a cytotoxic environment for the cells. Because we were able to optimize how much plasmin to use for degradation and for how long, we tested fixed samples for the potential degradation of the cellular layer on the microfibers. To ensure the cellular layer was not destroyed, we used confocal imaging to demonstrate that each layer was in tact as well as various ECM proteins. However, we were unable to fully degrade the fibrin core from the fibrin-alginate microfibers at longer

time points. We then chose fibrin-only microfibers for the remainder of the experiments because the degradation of the scaffold was much more apparent in comparison to the fibrin-alginate microfibers.

Once we found that cell and ECM layers remained intact after plasmin treatment, we moved to a live system to show the progression of degradation. These samples demonstrated that the scaffold could fully degrade in shorter culture time points. In addition, the first live cell culture degradation treatments on the scaffold wrapped around frames ensured that it was possible for the plasmin to pass through a thick cell layer and interact with the fibrin microfiber. Because we were able to degrade majority of the fibrin scaffold from microvasculature constructed, we determined that the cell construct could be moved to another system to progress closer to the goal.

After the live cell structures experiments, we moved to a new setup that gave us the ability to perfuse through the microfibers after degradation treatments. With this new system we were able to give a new foundation for the construction of the microvascular structures. As each different round of experiments passed, we made small changes from round to round to help improve the degradation process. The first experiment demonstrated that the degradation process was not robust enough in the three compartment device. This led to the following experiment where we began fibrinolysis on the outer compartments earlier than originally planned, at day two. We used an earlier degradation to try to create a smaller amount of the fibrin core that needed to be degraded later on in the experiment. Yet, this small step was still found to not be robust enough for full luminal formation and led to another suggestion; to shorten the length of the scaffold. Finally when the third round of experiments was done, we used the smallest possible

length of microfiber possible to help achieve full degradation. However when we were unable to create a lumen after beginning degradation at day two and used a smaller amount of the scaffold, we determined that it would be best to change to another set up. One possible reason that the degradation did not occur as smoothly as the structures on the frames is that the cellular layer was too thick; it could have inhibited the plasmin diffuse through the structure created. Another reason is that the fibrin degradation products had nowhere to go but within the newly formed lumen, and therefore we could not engineer a perfusable microvascular structure.

After demonstrating that full degradation was not possible in the three chamber device, even after the two modifications we changed a few important parts of the set up. The main change we made was to alter the microfiber shape. In collaboration with the Mao lab and their PhD candidate Brian Ginn, we fabricated a new shape for the scaffolds that were used for the rest of the study. We interchanged the original microfibers for a hollow tube shape to obtain direct perfusion instead of indirect. This shape did not have to rely on degradation treatments for luminal formation because this new shape already possessed one. Initially, we fabricated the coiled microfibers with coils of fibrin onto regular mandrels. However, when we found many leaking issues from this fabrication method, we switched to PTFE coated mandrels and used them along with sheets of fibrin to fabricate the hollow microfibers. We analyzed several different scaffold preparation methods. The first being the style of how the microfiber sheets are wrapped around the PTFE mandrel: longitudinal or coiled coil or a combination of the two. For initial experiments, we used coiled hollow microfibers due to the ease in fabrication. The second preparation method we chose was the dehydration process used after the hollow

microfibers were fabricated and frozen; vaporized, vaporized and lyophilized, and lyophilized. Initial experiments showed vaporized microfibers were not suitable because they were problematic when we tried to perfuse them. This is because during the vaporization process, the walls of the fibers began to puff up, creating blockages throughout and ultimately not creating a clear lumen. We tried to cannulate the scaffolds with a needle before rehydration and perfusion to clear a pathway. However, this did not help because either the microfibers would break when attempting cannulation or when we rehydrated the microfiber, the blocked lumen reappeared. Ultimately, lyophilized was chosen because when we rehydrated the microfibers, these scaffolds were able to perfuse media through with no issues.

Once the technique for hollow microfiber fabrication was chosen, the next important step we considered was whether it was possible to perfuse through the scaffold without any leaking. Two methods were attempted to ensure no leaking; surgical glue and sutures. The first that was attempted was surgical glue. From the beginning trials, we showed that the fiber needed to be rehydrated before the glue could be used. Otherwise the scaffold would use the liquid glue to hydrate itself. Another characteristic that should be noted is that even after rehydration, when the surgical glue was placed on the microfiber, it would become stiff and brittle from the glue. This itself was a problem because of the potential cytotoxic effects that could harm the cells; not giving the cells enough of a stable foundation to adhere and proliferate to instead of just adhering and dying.

When it was determined surgical glue was not the correct choice, we attempted to use sutures to prevent leaking from the scaffold. We determined that sutures were the

best choice because they allowed a tight seal between the needle that the hollow fiber was resting on and the microfiber itself, decreasing the chances for leaking in the scaffold. In addition to this we created a more minimal system to run in parallel to the three chamber device. This setup focused on the inner chamber where the structures were constructed and allowed for a direct perfusion of the structures. The one chamber device also allowed for many experiments to run parallel to each other, giving more time to troubleshoot and fix problems being faced during experimentation.

We conducted an initial experiment with ECFCs cultured on the coiled hollow microfiber five days to see if the deposition of ECM and ECFC alignment had changed. Additionally we attempted to perfuse through the structure created to show if perfusion without leaking was possible with the allotted time for cell growth. It was clear that the topography of the hollow microfiber had slightly changed the formation of the ECM and the cell alignment. Instead of the cells aligning around the microfiber in an organized fashion, the cells followed the ridges of each of the coils. Due to this, a non-uniform layer of cells and ECM was formed and ridges were seen. This can create potential issue for long term perfusion because surface topographic features, such as the ridges, can impact the behavior of the endothelial cells[26, 27]. To ensure that the topographic features of the hollow microfibers did not affect the wrapping of ECM proteins, we ran two more experiments in parallel of ECFCs for five days. These experiments demonstrated that the cells followed a similar style adhesion and alignment that was previously seen with the microfibers;. Additionally, from these experiments we were able to perfuse DMEM through the five day structure with minimal leaking. This encouraged for the progression to begin vSMC culture in addition to the ECFC culture to create a robust cellular layer.

Conclusion

This study demonstrates the progression of vessel development from a fibrin hydrogel microfiber to a perfusable vascular structure. Each step in the progression lead to another new accomplishment towards the final goal; from wrapping the fibers on frames, to setting up different devices for freestanding structures, to creating perfusable structures with no leaking. We were able to determine that it is possible to create vascular structures from the scaffold, with the tunica intima and media layers of the microvasculature clearly created and seen via confocal imaging. Additionally, the ECM deposition from the coculture is significantly larger than previously seen due to a longer culture period. This demonstrates that the device style experiments allowed the structures to not have as much contact while changing media, and therefore increasing the possibility of polymerization of ECM proteins. Finally, the last experiments in the study demonstrated that it is possible to obtain perfusable microvasculature using the single-chamber device and hollow microfiber shape.

For future experiments, first we will begin with the cocultures of ECFCs and SMCs for a twenty day experiment to create a thick cell layer. Once this cell layer is created, we will perfuse the microvasculature created with either a syringe or a peristaltic pump from anywhere between a few minutes to a few days. Other various adjustments could be considered to help create a stable system that results in the freestanding microvasculature we aim to construct. One possible alternative is to encapsulate plasmin within the fibrin microfibers to help increase the degradation rate. This adjustment would be used for both the microfibers and hollow shaped microfibers. Additionally, with this alternative, we would be able to use both microfiber shapes for both the three chamber and one chamber devices used throughout this study to achieve the maximum amount of

vascular structures. Another possible adjustment that could be made is to seed endothelial cells on the inside of the hollow microfibers while culturing vSMCs on the outside. We would then perfuse media through the hollow fibers in addition to the normal media changes in the system.

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Curriculum Vitae

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EDUCATION

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Bachelor of Science in Chemical and Biomolecular Engineering

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Minor in Entrepreneurship & Management

RESEARCH EXPERIENCE

Gerecht Lab

Johns Hopkins University, Baltimore, MD

Researcher

Oct 2011 – Present

Microvasculature Tissue Engineering Project

Sept 2013 – Present

- Conducts research pertaining to the construction of micro blood vessels from a starting foundation of a hydrogel biomaterial fiber
 - Modified a prototype that allows the formation of vascular structures. Once these structures are formed, prepared experiments to degrade the fiber so that the vascular structures are the only thing remaining.
 - Analyzed the role of the extracellular matrix in the formation of the structures
- Prepare experiments and analyze data from the experiments to compile a more comprehensive analysis

Research Assistant

Breast Cancer Tumorigenesis Project

Oct 2011 – Aug 2013

- Participated in research involved mostly with the influence of the extracellular matrix on vascular structures in tumorigenic breast cancer cells
 - Analyzed how hypoxia affects the structure of breast cancer cell-derived matrix. In addition, analysis gave further analysis on how hypoxic matrix may regulate angiogenesis in tumors
 - Analyzed the role of fibronectin in the extracellular matrix and how it can affect angiogenesis in tumors
- Prepared experiments and analyzed the resulting data to compile a more comprehensive analysis

EXTRACURRICULARS

JHU Chemical Engineering Graduate Student Liason Committee

Baltimore, MD

Masters Representative

September 2014 – Present

- Organize social, volunteering, and sporting activities for the Chemical Engineering Department graduate students
- Assist with set up, running, and clean up of events planned for the group
- Helped create a community within the Chemical Engineering Major

JHU Chapter of AIChE/SBE

Baltimore, MD

Member

Sept 2010 – May 2014

- Participates in biweekly chemical and biomolecular engineering events

Senior Representative

Sept 2013 - May 2014

- Promote AIChE/SBE and its events by making announcements in class to attract students to the group
- Assist with set up, running, and clean up of events planned for the group